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Fermentative Production of Cysteine by *Pantoea ananatis*

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ABSTRACT Cysteine is a commercially important amino acid; however, it lacks an efficient fermentative production method. Due to its cytotoxicity, intracellular cysteine levels are stringently controlled via several regulatory modes. Managing its toxic effects as well as understanding and deregulating the complexities of regulation are crucial for establishing the fermentative production of cysteine. The regulatory modes include feedback inhibition of key metabolic enzymes, degradation, efflux pumps, and the transcriptional regulation of biosynthetic genes by a master cysteine regulator, CysB. These processes have been extensively studied using *Escherichia coli* for overproducing cysteine by fermentation. In this study, we genetically engineered *Pantoea ananatis*, an emerging host for the fermentative production of bio-based materials, to identify key factors required for cysteine production. According to this and our previous studies, we identified a major cysteine desulfhydrase gene, *ccdA* (formerly PAJ_0331), involved in cysteine degradation, and the cysteine efflux pump genes *cefA* and *cefB* (formerly PAJ_3026 and PAJ_p0018, respectively), which may be responsible for downregulating the intracellular cysteine level. Our findings revealed that *ccdA* deletion and *cefA* and *cefB* overexpression are crucial factors for establishing fermentative cysteine production in *P. ananatis* and for obtaining a higher cysteine yield when combined with genes in the cysteine biosynthetic pathway. To our knowledge, this is the first demonstration of cysteine production in *P. ananatis*, which has fundamental implications for establishing overproduction in this microbe.

IMPORTANCE The efficient production of cysteine is a major challenge in the amino acid fermentation industry. In this study, we identified cysteine efflux pumps and degradation pathways as essential elements and genetically engineered *Pantoea ananatis*, an emerging host for the fermentative production of bio-based materials, to establish the fermentative production of cysteine. This study provides crucial insights into the design and construction of cysteine-producing strains, which may play central roles in realizing commercial basis production.

KEYWORDS *Pantoea ananatis*, amino acid fermentation, cysteine, cysteine desulfhydrase, cysteine efflux

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Cysteine is an important amino acid in the pharmaceutical, food, and cosmetic industries. Most major amino acids are commercially produced by bacterial fermentation, but cysteine is one of the few for which efficient fermentation methods are not yet available. At present, major industrial methods for producing cysteine include the hydrolysis of human hair or animal feathers and enzymatic conversion from DL-2-amino-Δ^2-thiazoline-4-carboxylic acid, a petroleum-derived raw material (1, and for a review, see reference 2). The bacterial fermentative production of cysteine is a major challenge in the amino acid fermentation industry because of its toxicity to cells (3, 4) and stringent metabolic regulation (for a review, see reference 5). To manage its deleterious effects, cells are equipped with several regulatory systems which facilitate

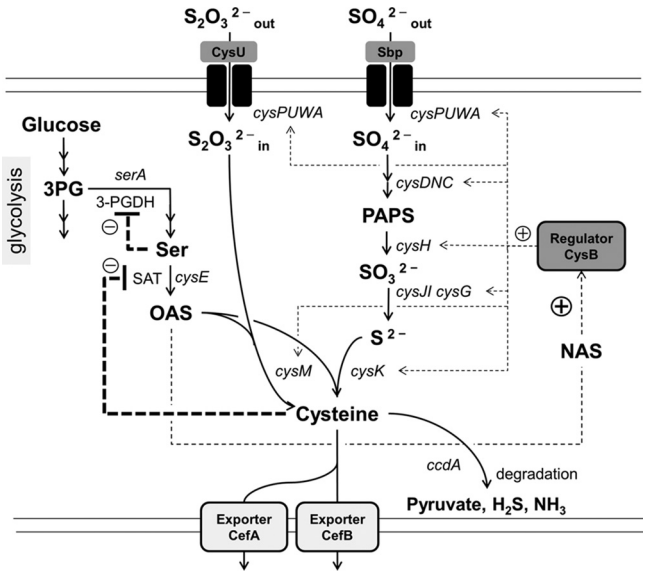

FIG 1 Biosynthetic pathway and regulation of cysteine synthesis in *Pantoea ananatis* and related bacteria. The major metabolites and enzymes responsible for cysteine biosynthesis from glucose and thiosulfate or sulfate are indicated by solid lines. The key regulatory effects provided by feedback inhibition of SAT (encoded by *cysE*) and 3-PGDH (encoded by *serA*) by cysteine and L-serine, respectively, as well as transcriptional regulation by the master regulator of sulfur metabolism, CysB, are indicated by dotted lines. NAS, *N*-acetylserine; OAS, *O*-acetylserine; PAPS, 3-phosphoadenosine 5-phosphosulfate; 3-PG, 3-phosphoglycerate; 3-PGDH, 3-phosphoglycerate dehydrogenase; SAT, serine acetyltransferase; Sbp, sulfate-binding protein; Ser, serine.

the strict control of intracellular cysteine levels. These regulatory mechanisms include feedback inhibition of serine acetyltransferase (SAT) and 3-phosphoglycerate dehydrogenase (3-PGDH), two key enzymes in cysteine biosynthetic pathways, by cysteine and serine, respectively (see Fig. 1 for details on the biosynthetic pathway and cysteine metabolism in *Pantoea ananatis* and related bacteria) (6–9). A master regulator of sulfur metabolism, CysB, controls the transcription of most genes involved in sulfur assimilation and cysteine metabolism. The CysB-mediated induction of sulfur assimilation genes promotes cysteine production by coordinating the flow of carbon and sulfur metabolites (10, 11). CysB is activated through its homotetramerization via the binding of *N*-acetylserine, a spontaneously occurring variant of *O*-acetylserine (5), thereby modulating the availability of carbon flow toward cysteine to coordinate the supply of sulfur. This regulatory mechanism controls the supply of cysteine at its biosynthetic level. The degradation of cysteine is another mode of regulation. In *Escherichia coli*, multiple cysteine desulfhydrases (CDs) catalyze the degradation of cysteine into pyruvate, ammonia, and sulfide (12, 13). In *P. ananatis*, a cysteine-inducible CD encoded by *ccdA* plays a central role in the degradation of cysteine (14). Other modes of regulation include the efflux of excess cysteine by a specific cysteine exporter, which has been proposed to function as a safety valve, where the cysteine efflux pump encoded by *cefA* in *P. ananatis* has been shown to possess this function (14).

The deregulation of these strict control systems is essential for overproducing cysteine in bacteria. Enhancing cysteine efflux pumps is also crucial for downregulating the intracellular level of cysteine to avoid any toxic effects and for secreting cysteine into the fermentation medium to facilitate further isolation processes (14–17). *E. coli* has been extensively studied as a host strain for the fermentative production of cysteine, where previous studies have identified core factors related to cysteine production, i.e., the deregulation of feedback loops by introducing a feedback inhibition-insensitive mutant for 3-PGDH (18) and SAT (19, 20), overexpression of cysteine efflux pumps (14–17, 21), and disruption of CDs (12, 13).

P. ananatis, a member of the *Enterobacteriaceae* family of bacteria, is an important research subject, which has been studied as a plant pathogen (for a review, see

reference 22). In recent years, nonpathogenic *P. ananatis* and closely related species have attracted interest in the fermentation industry because they are promising tools in metabolic engineering (23–25). Recent studies have indicated the potential of *P. ananatis* for the overproduction of a variety of useful chemicals, including amino acids and related compounds (L-glutamate [26], L-aspartate [27], and L-3,4-dihydroxyphenylalanine [28]), vitamins (pyrroloquinoline quinone [25], ascorbic acid intermediates [29], and vitamin E [30]), and other chemical compounds (2,3-butanediol [X. W. Jiang, 9 May 2007, Chinese Patent Office]). One of the greatest successes is in the development of the L-glutamate fermentation technology, where the characteristic growth of this microbe under acidic conditions (pH approximately 4) has facilitated the fermentative production of glutamate at an acidic pH, which is an environment where glutamate has low solubility, and thus, glutamate production is accompanied by its crystallization in culture (26, 31). The recent development of genetic recombination techniques and exploration of the complete genome sequence of *P. ananatis* have allowed its exploitation as a bacterial host for fermentation (23, 24).

In this study, we investigated the fermentative production of cysteine using *P. ananatis* AJ 13355 (23). We determined the effects of two previously identified genes encoding CD as a major cysteine degradation enzyme (*ccdA* product), and an efflux pump (*cefA* product), as well as another previously identified efflux pump (designated *cefB* [cysteine efflux], formerly PAJ_p0018), on cysteine fermentative production. We also demonstrated the overexpression of *O*-acetylserine sulfhydrylase B (OASS-B) encoded by *cysM* (32, 33), an identified bottleneck in the cysteine biosynthetic pathway, to elevate intracellular cysteine levels, where we found that its effects were modulated by combining the deletion of the degradation gene with the overexpression of efflux genes. This and our related study (34) are the first demonstrations, to our knowledge, of cysteine production in *P. ananatis*, and we demonstrated the significance of the deletion of its unique CD and the overexpression of efflux pumps using a combination of genes in the cysteine biosynthetic pathway.

RESULTS

Screening the *P. ananatis* genomic library for genes that confer cysteine resistance identified a novel gene involved in cysteine efflux. To identify *P. ananatis* genes that confer cysteine resistance when overexpressed, *E. coli* wild-type strain MG1655 was transformed with a multicopy genomic library prepared from *P. ananatis* genomic DNA, and cells were challenged on M9 minimal medium plates supplemented with 2 to 4 mM cysteine. These conditions showed that the toxicity of cysteine was sufficient to inhibit colony formation for up to 2 days. Colonies were selected after 2 days, and the genes that contributed to this phenotype were identified by sequencing the plasmid DNA. In our previous studies, we characterized the functions of two genes that conferred cysteine resistance. According to our results, one of the genes identified, *ccdA*, encodes a CD involved in cysteine degradation, and the other, *cefA*, encodes a cysteine efflux pump; both are functionally associated with cysteine resistance in *P. ananatis* (14). In the present study, we characterized another isolated gene, *cefB* (cysteine efflux; formerly PAJ_p0018), for which the coding protein possesses multiple predicted transmembrane helices (nine transmembrane helices predicted by SOSUI [35]) with a conserved motif characteristic of the drug/metabolite transporter superfamily (36), thereby suggesting that this gene encodes a novel cysteine efflux pump. Figure 2 shows the growth of *E. coli* wild-type strain MG1655 when challenged under conditions with a toxic level of cysteine (200 μM) in the M9 minimal medium. The overexpression of *eamA* (17), *leuE* (34), and *cefA*, known cysteine efflux pumps, and *ccdA*, a known cysteine decomposer, improved growth under these conditions (i.e., reduced the lag), which confirmed the validity of the experimental conditions and their effects in enhancing cysteine resistance. It should be noted that we cointroduced a transcriptional regulator, CefR, with CefA, because the expression of CefA is known to be dependent on this transcription factor, and the full function of CefA was achieved

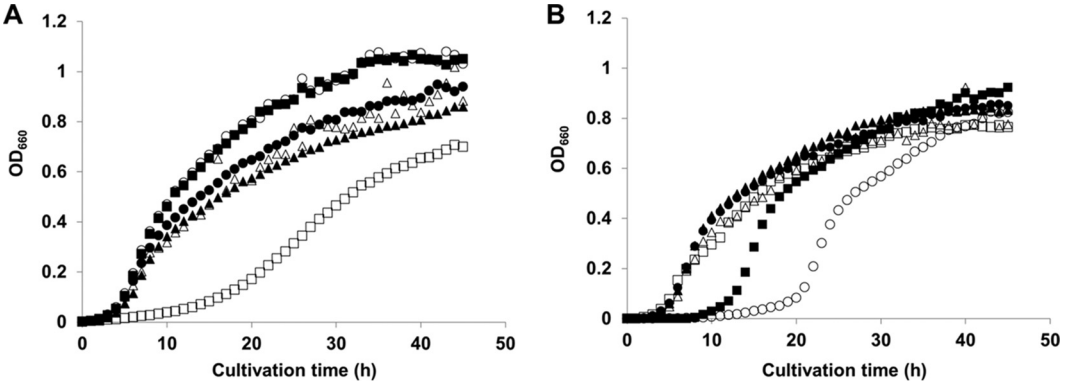

FIG 2 Resistance to cysteine. *Escherichia coli* MG1655 strains carrying each plasmid containing a series of genes identified as sources of cysteine resistance were challenged by 200 μM cysteine in M9 medium. Representative growth curves are shown for the strains carrying the vector (pSTV-29, ○), *leuE* (pSTV-leuE, ■), *eamA* (pSTV-eamA, □), *ccdA* (pSTV-ccdA, ●), *cefA* (pSTV-PA36ccd, △), and *cefB* (pSTV-cefB, ▲) without cysteine (A) and with 200 μM cysteine (B).

only when CefR was also introduced (14). The overexpression of *cefB* also conferred cysteine resistance, where the effect was comparable to that of the known efflux pumps, thereby supporting our hypothesis that the *cefB* product has a role in cysteine efflux. The effect observed with *leuE* was weaker, presumably due to the lower capacity of this transporter to export cysteine. An *E. coli*-based cysteine producer constructed using *leuE* instead of *eamA* exhibited significantly lower cysteine production, which reflected the difference in export capacity (G. Nonaka, unpublished data). *E. coli* strain MG1655 that overexpressed *eamA* exhibited significantly slower growth under conditions without added cysteine (data not shown), presumably because the capacity for cysteine export was too high to maintain a sufficient level of intracellular cysteine, although we have no experimental evidence to support this suggestion.

Overexpression of *cefB* increased the fermentative production of cysteine. To characterize the activity of *cefB* as a cysteine efflux pump and to explore its possible application in fermentative cysteine production, we overexpressed *cefB* in a model *P. ananatis* cysteine-producing strain. We constructed a model strain that carried a feedback-insensitive mutant *cysE* gene, i.e., *cysEX* encoding a mutant SAT (harboring an amino acid substitution, T167A) from *E. coli* (20), which has been widely utilized for cysteine production (21, 37, 38). We employed *cysEX* among the many known mutant alleles of *cysE* to replicate the experimental conditions used in a previous study of other cysteine efflux pumps, EamA and EamB (15, 39). *P. ananatis* strain SC17 (a derivative of AJ13355, which was selected as a low-mucus-producing mutant [31]) carrying *cysEX* on a plasmid was used as the host cysteine-producing (strain CYS1-1). Then, a second plasmid containing *cefB* was introduced to obtain the strain CYS1-2, where the gene was under the control of a constitutive strong promoter of the *nlpD* gene in *E. coli*, P_{nlp0} (34). It should be noted that strain SC17 itself was unable to produce detectable levels of cysteine in the media (data not shown), and therefore, a feedback-insensitive mutant *cysE* (i.e., *cysEX*) was required to produce a trace of cysteine. Strain CYS1-2 was aerobically cultivated in test tubes using a production medium supplemented with glucose as the main carbon source and thiosulfate as the main sulfur source, and we evaluated its ability to produce cysteine in the medium by analyzing the products, according to published methods (15, 17, 21), based on the method described by Gaitonde (40). The results indicated that the overexpression of *cefB* significantly increased cysteine production in the medium (Table 1). This property of cysteine overproduction, the phenotypic characteristic related to cysteine resistance, and the secondary structure typical of transporters are consistent with our hypothesis that the *cefB* product exhibits cysteine efflux activity, which may be effective for the fermentative production of cysteine.

Deletion of *ccdA* increased the fermentative production of cysteine in the culture medium. To investigate the importance of the major CD in *P. ananatis* for

TABLE 1 Effects of *cefB* and *ccdA* on cysteine production in *Pantoea ananatis* model producers[a]

Strain by expt	Genotype or gene(s) on plasmids[b]	Cysteine production (mg · liter^{-1})	OD_{600}	% cysteine yield (g/g)
Expt 1[c]				
CYS1-1[d]	*cysEX*, vector	95 ± 6	4.3 ± 0.2	0.26 ± 0.04
CYS1-2	*cysEX cefB*	209 ± 16	9.3 ± 0.2	0.36 ± 0.01
Expt 2[e]				
CYS2-1	*cysE5*, vector	169 ± 4	27.8 ± 0.5	0.28 ± 0.01
CYS2-2	*cysE5 ccdA*	71 ± 5	23.5 ± 0.3	0.12 ± 0.01
Expt 3[f]				
CYS3-1	WT, *cysE5*	202 ± 14	34.9 ± 0.6	0.34 ± 0.02
CYS3-2	*ccdA*::*kan cysE5*	516 ± 46	34.0 ± 1.5	0.84 ± 0.06

[a]Values represent the averages ± the standard deviation based on the results from four independent experiments.
[b]WT, wild type.
[c]Sixty grams per liter glucose was supplied (instead of 40 g · liter^{-1}; see standard fermentation medium in the Materials and Methods). Cultivation was terminated at 18 h after inoculation.
[d]Residual glucose (20.3 ± 1.3 g · liter^{-1}) was detected.
[e]Cultivation of CYS2-1 and CYS2-2 was terminated at 23 h and 25 h after inoculation, respectively.
[f]Cultivation of CYS3-1 and CYS3-2 was terminated at 19 h after inoculation.

cysteine production and to explore its possible applications in fermentative cysteine production, we overexpressed and deleted *ccdA* in a model *P. ananatis* cysteine-producing strain (CYS2-1 and CYS3-1 for overexpression and deletion, respectively) carrying the feedback-insensitive mutant *cysE* gene, i.e., *cysE5* encoding a mutant SAT (containing amino acid substitutions V95R and D96P) from *E. coli* (19). We observed strong negative effects on the production of cysteine when *ccdA* was overexpressed by use of pACYC-ccdA (strain CYS2-2), which is consistent with its functional characteristic as a dominant CD with a major role in cysteine degradation (Table 1). We also observed significant positive effects on cysteine production when *ccdA* was deleted from the producer strain (CYS3-2), thereby indicating the importance of its absence from the host strain for cysteine production (Table 1).

Induction of CD activity exhibited by CcdA negatively affected the production of cysteine in a high-level producer strain. We investigated the significance of cysteine efflux and degradation for cysteine production via the application of the novel efflux pumps *cefA* and *cefB* and the CD *ccdA* in *P. ananatis* using a high-level cysteine producer. We constructed a *P. ananatis*-based producer strain, AG4854 (engineered from SC17), for further analysis. This strain AG4854 was deregulated with respect to two important key enzymes, SAT and 3-PGDH, by introducing genes encoding the feedback resistant mutants *cysE5* (19) from *E. coli* harboring amino acid substitutions V95R and D96P, and *serA348* (18) from *P. ananatis* harboring an amino acid substitution, N348A. We employed the mutant allele *cysE5* instead of *cysEX* because it had advantages in terms of its enzymatic parameters *in vitro* as well as its growth phenotypes when introduced into the producer strains (see the Discussion for further details). The AG4854 also overexpressed the efflux pump LeuE from *E. coli* (encoded by *leuE*, originally identified as an L-leucine efflux pump [41] which exhibits cysteine efflux activity [34]) and an enhanced thiosulfate transporter, CysPUWA (encoded by *cysPUWA*), by promoter replacement. CysPUWA is a bottleneck in the intracellular sulfur supply pathway (42). The target genes were driven by constitutive strong promoters of either *ompC* (for *cysE5*; denoted P_{ompC}) or *nlpD* (for *serA348*, *leuE*, and *cysPUWA*; denoted P_{nlp8}) from *E. coli* (see Materials and Methods and a previous study [34] for details on the constructs). This engineered producer strain AG4854 produced approximately 1.3 g · liter^{-1} cysteine (Fig. 3).

To investigate the bottleneck in cysteine production using AG4854 and to achieve greater production based on this strain, we introduced most genes responsible for cysteine biosynthesis (i.e., *cysPUWA*, *serA*, *serB*, *serC*, *cysE5*, *cysK*, *cysM*, and *leuE*) and

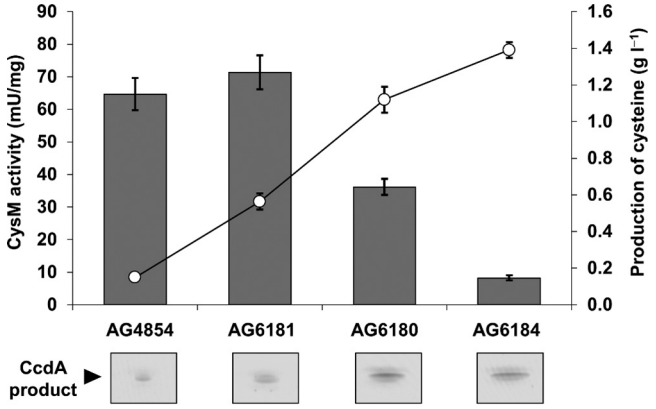

FIG 3 Effects of CysM overexpression on CcdA activation and cysteine production. The production of cysteine in the medium (gray bars) and activity of CysM (*O*-acetylserine sulfhydrylase B) based on the crude cell extract (white circles) from each CysM-enhanced variant (AG6181, AG6180, and AG6184) constructed by engineering cysteine-producing *Pantoea ananatis* strain AG4854 are shown (top). The images of native PAGE and activity staining of the *ccdA* product obtained from the crude cell extract of each of the CysM variants are shown (bottom). Values represent the averages based on the results from four independent experiments, and the error bars represent one standard deviation.

selected effective genes (data not shown). Among the genes tested, we found that *cysM*, encoding OASS-B (see Fig. 1 for the cysteine biosynthetic pathway), increased the production of cysteine, thereby suggesting that this is the bottleneck for cysteine production by this strain (Fig. 3). It should be noted that *cysK* (encoding OASS-A, an isozyme of CysM) failed to increase the production of cysteine in our bottleneck screening (data not shown), suggesting that the synthesis of cysteine was carried out from thiosulfate supplied in the productive medium. Both CysM and CysK synthesize cysteine from sulfate, whereas only CysM synthesizes cysteine from thiosulfate (thiosulfate serves as a ready-to-use sulfur source for the synthesis of cysteine). Next, we constructed variants with different CysM expression levels based on the cysteine producer AG4854 via mini-Mu-mediated integration of *cysM* gene copies from *E. coli*, driven by variants of the constitutive *nlpD* promoter from *P. ananatis*. The relative activities of CysM in each variant strain (AG4854, AG6181, AG6180, and AG6184) were measured by assaying OASS activity in a crude cell extract using thiosulfate as a sulfur substrate, as shown in Fig. 3. Cysteine production peaked when a moderate increase in CysM activity was provided (Fig. 3; AG6181), and an additional increase in CysM activity severely inhibited production (Fig. 3; AG6180 and AG6184). We assumed that this negative effect was mainly associated with the degradative activity of CD encoded by *ccdA*, because this CD is cysteine inducible (14), and it may play a major role in controlling the intracellular cysteine concentration by ensuring that it remains below the threshold for toxicity.

To further confirm the relevance of the connection between CcdA and the CysM-mediated decrease in cysteine production, we determined the relative activity of CcdA in strains with altered CysM expression levels using an activity stain for CcdA in a crude cell extract separated by native PAGE (Fig. 3; also see Fig. S1A for original full gel image). The results indicated that the activity of CcdA increased in response to the activity of CysM, where a major increase in CcdA activity was correlated with a major drop in cysteine production (comparison of AG6181 with AG6180), which supported our hypothesis. There were no visible stains of CDs except the stains of CcdA (Fig. S1A), supporting that CcdA might be the only major contributor affecting the production of cysteine. To obtain further evidence based on quantitative methods, we measured and compared the cellular CD activity of crude extracts obtained from the strains with and without *ccdA* across the CysM variations (Table 2). The results reproduced the trend of the activity stain, where a major increase in CD activity across the CysM variants in the presence of *ccdA* was consistent with that of the CcdA stain (compare with intensity of

TABLE 2 Total cellular CD activity of the producer stains with CysM variations

Strain	*ccdA*	CD activity (avg ± SD) (mU)[a]
AG4854	WT	1.0 ± 0.07
AG6181		1.2 ± 0.12
AG6180		4.1 ± 0.40
AG6184		3.3 ± 0.41
AG4854-D	*ccdA*::*kan*	1.0 ± 0.03
AG6181-D		0.9 ± 0.09
AG6180-D		0.9 ± 0.18
AG6184-D		1.1 ± 0.04

[a]From three independent experiments. 1 U = 1 μmol/min/mg of protein.

bands in Fig. 3; also see Fig. S1B for its semiquantification values). The CysM-mediated induction of CD activity in the presence of *ccdA* disappeared in the absence of *ccdA* (Table 2), suggesting that the CcdA was the only inducible and thus served as the major CD during cysteine production, which might have negatively affected the production of cysteine when the activity of CysM was elevated.

Modulation of efflux and degradation in a high-level producer strain demonstrated the essential properties of these factors for efficient cysteine production. To investigate the effects of *ccdA* deletion on the production of cysteine, we evaluated the production and toxic effects of putative intracellular cysteine accumulation using a series of cysteine-producing strains with various levels of CysM. We observed increased cysteine production by the deletion of *ccdA* in all CysM-overexpressing backgrounds, thereby suggesting that the cysteine-degrading activity of CcdA was significant when *ccdA* was present under the CysM-overexpressing conditions. Thus, deletion of *ccdA* was crucial for improving cysteine production by these engineered strains (Table 3). The growth (optical density [OD]) and metabolic activity (capacity for consuming glucose as a substrate) of the strains that lacked *ccdA* were negatively correlated with the activity of CysM (Table 3; OD at 600 nm [OD_{600}] and sugar consumption capacity were lower in all strains when *ccdA* was absent) determined in the previous experiment (see Fig. 3 for the CysM activity), which supported our hypothesis that CysM contributes to an enhanced production capacity in cells and eventually had negative effects on cysteine production when *ccdA* was present and on cell growth when *ccdA* was absent. In particular, the strains with higher CysM activity (AG6184) produced more cysteine when *ccdA* was absent (compared with that when present), and they were capable of consuming only half of the sugar, while the OD increased only by half compared to strains with *ccdA*.

Next, we tested whether the novel efflux pumps CefA and CefB could export cysteine from the engineered producer strains with CysM overexpression, thereby eliciting positive effects on cysteine production as well as avoiding negative effects on growth caused by the CysM-mediated elevated level of intracellular cysteine. The producer AG6184, a strain with poor growth due to high CysM activity, was employed

TABLE 3 Effects of *ccdA* deletion on the fermentative production of cysteine[a]

Strain	*ccdA*	Cysteine production (mg · liter^{-1})	OD_{600}	% cysteine yield (g/g)	Cultivation time (h)[b]
AG4854	WT	1,246 ± 42	17.4 ± 0.3	3.1 ± 0.1	<16
AG6181		1,384 ± 54	16.5 ± 0.8	3.5 ± 0.1	18–20
AG6184		169 ± 4	12.6 ± 1.0	0.4 ± 0.0	21–21.5
AG4854-D	*ccdA*::*kan*	1,254 ± 273	16.3 ± 0.5	3.1 ± 0.7	17.5–18.5
AG6181-D		1,576 ± 144	15.1 ± 0.5	3.9 ± 0.4	20–21
AG6184-D		283 ± 39	6.3 ± 0.1	1.6 ± 0.4	>21.5[c]

[a]Values represent averages ± the standard deviation based on the results from based on four independent experiments.
[b]Cultivation was terminated when 40 g · liter^{-1} of glucose was consumed.
[c]Residual glucose (22.1 ± 1.4 g · liter^{-1}) was detected.

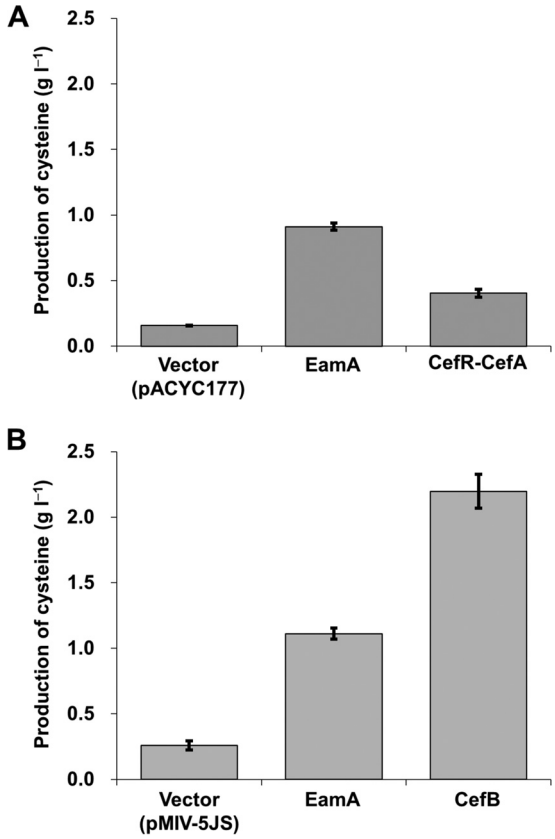

FIG 4 Effects of enhancing the efflux pumps on the fermentative production of cysteine in an engineered producer strain AG6184. Each gene encoding the cysteine efflux pumps was introduced into AG6184 using pACYC177 (vector) driven by the original promoters (A) or pMIV-5JS (vector) driven by P_{nlp0} (B). Productive cultivation was performed under standard conditions and using standard medium, except that glucose was supplemented at 60 g · liter^{-1} instead of the usual 40 g · liter^{-1}, and cultivation was terminated at 28 h when approximately 40 g · liter^{-1} glucose had been consumed (within approximately 10% of the error range) (A), or under standard conditions and using standard medium with regular 40 g · liter^{-1} glucose, and termination of cultivation at 22 h when 40 g · liter^{-1} glucose had been consumed (within approximately 10% of the error range) (B). A transcriptional regulator, *cefR*, was cointroduced with *cefA* by use of pACYC-PA36ccd to completely enhance the effects of *cefA*. Values represent the averages based on the results from four independent experiments, and the error bars represent one standard deviation.

as a host in this evaluation. We found that a well-established cysteine efflux pump, EamA, and all novel efflux pumps successfully rescued poor cysteine production (Fig. 4), thereby indicating that these efflux pumps were active in these high-level producer strains with CysM overexpression. Thus, the combination of CysM with the efflux pumps synergistically increased cysteine production, which supports the hypothesis that CysM contributes to increasing intracellular cysteine levels while the efflux pumps export the consequent high accumulation of cysteine across the membrane. We introduced the transcriptional regulator CefR together with CefA because the expression of CefA depends on this transcription factor, and the full function of CefA is achieved only when CefR is also introduced (14). This experiment lacked a quantitative assessment of each efflux pump to determine the capacity for cysteine production, because fine-tuning of the expression level was necessary to exploit their full ability, but we still performed a qualitative assessment of the ability of the efflux pumps to contribute to cysteine production.

DISCUSSION

In this study, we demonstrated the fermentative production of cysteine in *P. ananatis* AJ 13355. We confirmed the effects of two previously characterized genes that

encode CD, a major cysteine degradation enzyme (encoded by *ccdA*), and an efflux pump (encoded by *cefA*), as well as an additional efflux pump (encoded by *cefB*) characterized in the present study, on the fermentative production of cysteine. We demonstrated the increase in CysM activity, an identified bottleneck in the cysteine biosynthetic pathway, whose overexpression presumably contributes to elevated intracellular cysteine levels, as well as its effects when combined with the deletion of the degradative gene or in modulating the expression of the efflux gene. According to our findings, deletion of the major cysteine degradative gene *ccdA* and overexpression of the cysteine efflux genes *cefA* and *cefB* were crucial factors that affected the establishment of fermentative cysteine production in *P. ananatis*, and they were also key factors for obtaining a higher cysteine yield when combined with genes (e.g., *cysM*) in the cysteine biosynthetic pathway. According to our findings, the production of approximately 2.2 g · liter^{-1} was obtained as the highest titer (Fig. 4B), which is fairly encouraging compared with that of 2.3 g · liter^{-1} using *E. coli* (43). We expect that stepwise genetic engineering to fine-tune the crucial factors identified in this study, together with new bottlenecks on the cysteine biosynthetic pathway, will steadily increase the production of cysteine by *P. ananatis*.

P. ananatis is one of the most promising emerging hosts for the fermentation of amino acids and related compounds, and it may have many advantages for cysteine fermentation compared with *E. coli* and other microbes. First, managing the degradation activity of cysteine is considered to be one of the most important aspects of its production, and *P. ananatis* is beneficial because it possesses a rather specific and simplified system for cysteine degradation. In our previous study, we identified CcdA as the only major CD in *P. ananatis* (14), and the present study demonstrated that its simple deletion drastically altered the productivity and cytotoxicity of cysteine, thereby identifying CcdA as an essential factor for the establishment of cysteine fermentation in this organism. The deletion of CDs in *E. coli* was also effective for cysteine production, but this microorganism encodes several major CDs, and strains with multiple gene knockouts still exhibit significant cellular CD activity (12). Thus, some genes may still need to be identified as mediators of CD activity, and current knowledge is insufficient for eliminating the entire activity to achieve complete protection from degradation. Moreover, all known CDs in *E. coli* have primary functions as enzymes in other cellular processes, so the deletion of these genes may have negative effects. The OASSs CysK and CysM catalyze the reaction of *O*-acetyl-L-serine with sulfide to produce cysteine (32, 33). The cystathionine β-lyase family members MetC and MalY cleave the Cβ-S bond of diverse substrates to convert cystathionine into homocysteine (44, 45). The tryptophanase TnaA catalyzes the hydrolysis of L-tryptophan to indole, pyruvate, and ammonia (46).

The efflux of cysteine from cells via transporters is also important for its production, and *P. ananatis* has a rather specific system for transporting cysteine out of cells. In our previous study, we identified the first known cysteine-inducible efflux pump for cysteine, CefA, whose physiological function is closely associated with controlling cellular cysteine levels when cysteine is in excess (14). Further characterization of the entire transport system of CefA and CefB is required, including its substrate specificity and other important kinetic factors as a transporter, as well as its optimal expression levels. Managing unwanted production due to leakage via a transporter as a consequence of relaxed substrate specificity and its fine-tuned expression levels to prevent the disturbance of important cellular processes are general challenges in the development of amino acid fermentation (47). Finally, *P. ananatis* is more tolerant to environmental stresses than *E. coli*, particularly acidic conditions (23, 31). Fermentation under acidic conditions is beneficial in reducing the amount of alkali to improve cost-effectiveness and avoiding spontaneous oxidation under aerobic fermentation conditions.

It should be noted that the different alleles of the SAT mutant exhibited distinctive growth phenotypes. Strains with *cysEX* (20) had remarkably lower OD_{600} values than those with *cysE5* (19) (compare the OD_{600} values in Table 1). Further enzymatic characterization of each allele is required to completely understand the mechanisms

TABLE 4 Oligonucleotides used for the construction of plasmids

Plasmid name	Vector	Restriction site	Gene/ promoter	Source	Primer sequence (5′ to 3′)
pMIV-P_{nlp0}	pMIV-5JS	SalI/PaeI	P_{nlpD}	*E. coli*	AGCTGAGTCGACCCCCAGGAAAAATTGGTTAATAAC
					AGCTGAGCATGCTTCCAACTGCGCTAATGACGC
pMIV-P_{nlp4}	pMIV-5JS	SalI/PaeI	P_{nlpD}	*P. ananatis*	AGCTGAAAGCTTGCATGCACGCGTGGCGATCTGGCCTGACTGC
					AGCTGAGTCGACCCCGTGGTGGCAACCTTTAAAAAACTG
pMIV-P_{nlp0}-cefB	pMIV-P_{nlp0}	SalI/XbaI	*cefB*	*P. ananatis*	CCGTCGACATGAACGCATTACTCTATGC
					AATCTAGATTATAGCGTGCCCGGCATGGG
pMIV-P_{nlp23}-cefB	pMIV-P_{nlp23}	SalI/XbaI	*cefB*	*P. ananatis*	CCGTCGACATGAACGCATTACTCTATGC
					AATCTAGATTATAGCGTGCCCGGCATGGG
pMIV-P_{nlp0}-eamA	pMIV-P_{nlp0}	SalI/XbaI	*eamA*	*E. coli*	ACGCGTCGACATGTCGCGAAAAGATGGGGGTG
					CTAGTCTAGATTAACTTCCCACCTTTACCGC
pMIV-P_{nlp4}-cysM	pMIV-P_{nlp4}	SalI/XbaI	*cysM*	*E. coli*	AGCTGAGTCGACGTGAGTACATTAGAACAAACAA
					AGCTGATCTAGAAGTCTCCGATGCTATTAATCC
pMIV-P_{nlp1}-cysM	pMIV-P_{nlp1}	SalI/XbaI	*cysM*	*E. coli*	AGCTGAGTCGACGTGAGTACATTAGAACAAACAA
					AGCTGATCTAGAAGTCTCCGATGCTATTAATCC
pSTV-leuE	pSTV29	BamHI	*leuE*	*E. coli*	CGCGGATCCAGTGGTCATTTAGTGC
					CGCGGATCCTGTGGGATTTGAAGCATCC
pSTV-cefB	pSTV29	BamHI	*cefB*	*P. ananatis*	CGCGGATCCTGTTTACGGTAATCCTGTC
					CGCGGATCCACAAGTGCAGGGCTTTC

involved, but the properties of SAT might be an important determinant of carbon distribution in the glycolysis and serine/cysteine node at 3-phosphoglycerate, presumably by altering the concentration of serine, which eventually reduces the activity of 3-PGDH (Fig. 1 shows the pathways). According to the growth characteristics determined in the present study and the *in vitro* enzymatic characteristics in terms of inhibition by cysteine (19, 20), we propose that *cysE5* is more advantageous than *cysEX* when introduced into cysteine-producing strains.

In this study, we obtained important insights into the possible fermentative production of cysteine using *P. ananatis*. The two fundamental mechanisms that control degradation and efflux have been identified and clarified, so the next step is their application in the construction of actual producer strains for industrial use. In particular, the next requirements are selecting efflux pumps from among the available candidates and implementing their fine-tuned expression to manage any negative effects (e.g., growth, substrate consumption rate, and by-products), thereby allowing the maximum production of cysteine. After completing the fine-tuning of efflux and degradation, a routine step-by-step approach can be developed for the biosynthesis of cysteine in an effective manner. Thus, the fine-tuning of CysM is the next step. Maximizing production and scaling up will be further challenges. The results of the present study will play an important role in implementing the commercial production of cysteine-related compounds.

MATERIALS AND METHODS

Bacterial strains and plasmids. The primers used to construct the plasmids are listed in Table 4. The strains and plasmids used in this study are listed in Table 5. A gene deletion of *ccdA* in *P. ananatis* was performed according to the system based on Red-driven integration developed by Katashikina et al. (24, 48). To amplify a DNA cassette designed for inducing gene deletion, pMW-(λattL-Km^r-λattR) (kanamycin marker) was used as the template, and primer-1 (5′-CCGTGTCTGAAGCCTATTTTGCCCGCCTGCTGGGCTTGCCTTTTATTGCCTGAAGCCTGCTTTTTTATACTAAGTTGGCA-3′) and primer-2 (5′-CCGTGTCTGAAGCCTATTTGCCCGCCTGCTGGGCTTGCCTTTTATTGCCTGAAGCCTGCTTTTTTATACTAAGTTGGCA-3′) containing 50-nucleotide (nt) sequences homologous to the target region at the 5′ end of the chromosome were used as primers. The resulting PCR-generated DNA cassette contained a kanamycin resistance gene flanked by each 50-nt fragment homologous to the target genomic locus, which allowed homologous recombination by replacing the target gene with the kanamycin marker. Using electroporation, the fragment was introduced into *P. ananatis* SC17(0) harboring the plasmid RSF-Red-TER, which is a strain designed for inducing Red-driven recombination (24), and the transformants were selected using an antibiotic marker (donor strain). The chromosomal DNA prepared from the donor strain using the PurElute bacterial genome kit (EdgeBio, Gaithersburg, MD) was introduced into the target (recipient) strain by electroporation to induce the chromosomal transformation of Δ*ccdA*::*kan* by homologous recombination. The resulting target strains containing Δ*ccdA*::*kan* (typically 10 to 50 colonies could be obtained) were selected on kanamycin-containing plates.

TABLE 5 Strains and plasmids

Strain or plasmid	Description[a]	Source or reference[b]
Strains		
E. coli MG1655	Wild-type *E. coli* MG1655 (ATCC 47076)	ATCC
P. ananatis		
SC17	A low-mucus-producing mutant derived from wild-type *P. ananatis* AJ13355	NITE
SC17-D	SC17 Δ*ccdA*::Km^r	14
AG4854[c]	Cysteine production strain; *cysE5* and *leuE* (Mu) under the control of the *ompC* promoter and P_{nlp8}, respectively, and *serA348* (Mu) under the control of the P_{nlp8} were integrated, and the promoter of *cysPUWA* was replaced by P_{nlp8} in the genome of SC17	34[c]
AG6181	CysM-enhanced variant; *cysM* (Mu) under the control of P_{nlp1} was integrated in the genome of AG4854	This study
AG6180	CysM-enhanced variant; *cysM* (Mu) under the control of P_{nlp1} was integrated in the genome of AG4854	This study
AG6184	CysM-enhanced variant; *cysM* (Mu) under the control of P_{nlp4} was integrated in the genome of AG4854	This study
AG4854-D	AG4854 Δ*ccdA*::Km^r	This study
AG6180-D	AG6180 Δ*ccdA*::Km^r	This study
AG6181-D	AG6181 Δ*ccdA*::Km^r	This study
AG6184-D	AG6184 Δ*ccdA*::Km^r	This study
CYS1-1	SC17 harboring pACYC-E1 and pMIV-5JS	This study
CYS1-2	SC17 harboring pACYC-E1 and pMIV-cefB	This study
CYS2-1	SC17 harboring pMIV-cysE5 and pACYC177	This study
CYS2-2	SC17 harboring pMIV-cysE5 and pACYC-ccdA	This study
CYS3-1	SC17 harboring pMIV-cysE5	This study
CYS3-2	SC17-D harboring pMIV-cysE5	This study
SC17 (0)	Mutant resistant to λ Red recombinase selected from SC17	24
Plasmids		
pMIV-5JS	Cloning vector; SC101 *ori*; Cm^r	49
pMIV-$P_{nlp(x)}$	P_{nlpD} promoter variants (x = 0, 4, or 1) cloned into pMIV-5JS with T_{rrnB} (*rrnB* terminator)	This study
pMIV-cefB	*cefB* cloned into pMIV-P_{nlp0}	This study
pMIV-eamA	*eamA* from *E. coli* cloned into pMIV-P_{nlp0}	This study
pMIV-cysE5	*cysE5* (*E. coli*) under the control of the *ompC* promoter in pMIV-5JS	55
pMIV-P_{nlp4}-cysM	*cysM* from *E. coli* cloned into pMIV-P_{nlp4}	This study
pMIV-P_{nlp1}-cysM	*cysM* from *E. coli* cloned into pMIV-P_{nlp1}	This study
pSTV29	Vector, p15a *ori*, Cm^r empty vector	TaKaRa Bio
pSTV-eamA	*eamA* from *E. coli* cloned into pSTV29	14
pSTV-leuE	*leuE* from *E. coli* cloned into pSTV29	This study
pSTV-ccdA	*ccdA* from *P. ananatis* cloned into pSTV29	14
pSTV-cefB	*cefB* from *P. ananatis* cloned into pSTV29	This study
pSTV-PA36ccd	*cefA-cefR* from *P. ananatis* cloned into pSTV29	14
pACYC177	Vector, p15a *ori*, Km^r empty vector	Nippon Gene
pACYC-E1	*cysEX* (*E. coli*) cloned into pACYC184 under the control of *ompA* promoter	This study
pACYC-ccdA	*ccdA* from *P. ananatis* cloned into pACYC177	14
pACYC-EamA	*eamA* from *E. coli* cloned into pACYC177	14
pACYC-PA36ccd	*cefA-cefR* from *P. ananatis* cloned into pACYC177	14
pMH10	pACYC177 derivative harboring Mu-phage A and B genes encoding Mu transposase, *cts62* gene encoding Mu repressor, and the λ phage repressor gene *cI857*; Km^r	51
pMW-(λattL-Km^r-λattR)	Donor $attL_\lambda$-Km^r-$attR_\lambda$ cassette; Ap^r Km^r	24
RSF-Red-TER	λ *gam*, *bet*, and *exo* genes under the control of the P-element; *sacB* gene; Cm^r	24

[a]Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; (Mu), gene integration by Mu phage transposition-base system.
[b]ATCC, American Type Culture Collection; NITE, National Institute of Technology and Education.
[c]AG4854 was described in this reference as strain EYPS1976(s).

In this study, a constitutive promoter, P_{nlpD}, was used, which contained approximately 300 bp and 180 bp upstream of the *nlpD* gene obtained from the genomes of *E. coli* (locus tag b2742) and *P. ananatis* (locus tag PAJ_2318), respectively. These fragments were amplified using the primers listed in Table 4 and the genome of MG1655 or SC17 as the template. To obtain variants, the −10 and −35 regions of the original *nlpD* promoter, denoted P_{nlp0}, derived from *E. coli* were randomized, and thus, two derivative promoters were obtained, P_{nlp8} and P_{nlp23} (34). In addition, the Shine-Dalgarno (SD) sequence of the original *nlpD*, denoted P_{nlp4}, derived from *P. ananatis* was randomized, and thus, a derivative SD sequence was obtained, P_{nlp1} (34). The sequences of these promoters and SDs are shown in Table S1. The fragments containing promoter and SD sequence were inserted into the plasmid pMIV-5JS (49) using SalI and PaeI sites, thereby obtaining plasmids with the variant P_{nlpD}, pMIV-P_{nlp0}, pMIV-P_{nlp8}, pMIV-P_{nlp23}, pMIV-P_{nlp4}, and pMIV-P_{nlp1}.

Variants of OASS-B encoded by *cysM* were constructed using the Mu phage transposition-based integration system (50). The *cysM* gene in *E. coli* was amplified using the primers listed in Table 4, with genomic DNA from MG1655 as the template, and were then cloned into the plasmids pMIV-P_{nlp4} and pMIV-P_{nlp1} to generate pMIV-P_{nlp4}-cysM and pMIV-P_{nlp1}-cysM, respectively. The plasmids containing the P_{nlp}-driven *cysM* gene and chloramphenicol resistance marker flanked by the attachment sites of the Mu phage, designed for inducing mini Mu-mediated integration, were introduced by electroporation into strain SC17, which harbored the helper plasmid pMH10 (51) that expressed Mu transposase. Mu-mediated chromosomal integration was induced according to previously described methods (34, 52). Strains with the chromosomal P_{nlp}-*cysM* cassettes were selected on an agar plate containing chloramphenicol. The chromosomal DNA samples prepared from the SC17-based OASS-B variants were introduced into the recipient strain (AG4854) by electroporation to induce chromosomal transformation of the P_{nlp}-*cysM* cassettes by homologous recombination. The resulting AG4854-based OASS-B variants containing P_{nlp}-*cysM* cassettes in their chromosomes were selected using a chloramphenicol marker (AG6180, AG6181, and AG6184).

To construct the plasmid pACYC-E1, harboring *cysEX* (20), which encodes a mutant SAT from *E. coli*, pACYC-DE1 (K. Takumi and G. Nonaka, 1 January 2014, European Patent Office), carrying *eamA* and *cysEX* under the control of the *ompA* promoter, was digested with MnuI and self-ligated to delete an approximately 330-bp region inside the *eamA* gene.

Growth conditions. All strains were grown in Luria-Bertani (LB) (53), M9 minimal (53), or fermentation [15 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 0.1 mg of thiamine hydrochloride, 1.7 mg of $FeSO_4 \cdot 7H_2O$, 0.15 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.7 mg of $CoCl_2 \cdot 6H_2O$, 1.6 mg of $MnCl_2 \cdot 4H_2O$, 0.3 mg of $ZnSO_4 \cdot 7H_2O$, 0.25 mg of $CuSO_4 \cdot 5H_2O$, 0.6 g of tryptone, 0.3 g of yeast extract, 0.6 g of NaCl, 20 g of $CaCO_3$, 135 mg of L-histidine·HCl·H_2O, 4 g of $Na_2S_2O_3$, 2 mg of pyridoxine hydrochloride, and 40 g of glucose per liter] medium at 37°C (*E. coli*) or 34°C (*P. ananatis*), unless otherwise stated. The medium was supplemented with 20 $\mu g \cdot ml^{-1}$ kanamycin and/or 25 $\mu g \cdot ml^{-1}$ chloramphenicol, as required.

Screening and growth assay for cysteine resistance. Screening of genes that conferred cysteine resistance was performed as previously described (14). In the growth assay for cysteine resistance, each overnight culture cultivated in a test tube containing 3 ml of the M9 minimal medium was diluted to 1:100 with fresh M9 minimal medium containing 50 μM cysteine to obtain a total volume of 3 ml and was then grown overnight in test tubes with agitation. The cells were then inoculated into 4 ml of fresh M9 minimal medium in test tubes containing 0 or 200 μM cysteine to obtain an initial optical density at 600 nm (OD_{600}) of 0.006. Growth (OD_{660}) was automatically monitored in a TN-1506 incubator (Advantec Toyo, Tokyo, Japan).

Activity staining for CD. An overnight culture of each strain in the LB medium was diluted 1:25 with 50 ml of fermentation medium and grown aerobically for 4 h until logarithmic phase at 32°C before harvesting by centrifugation. The cells were washed three times with a wash buffer containing 10 mM Tris-HCl (pH 8.6), 100 μM dithiothreitol, and 10 μM pyridoxal phosphate (PLP) and were then resuspended in the wash buffer. Each crude cell extract was prepared by ultrasonicating the cell suspension and separating it by centrifugation. Proteins in the cell extracts (4 μg of total protein per sample) were mixed with a 5× loading buffer containing 10 mM Tris-HCl (pH 8.6), 30% glycerol, and 0.005% bromophenol blue and were separated by native polyacrylamide gel electrophoresis (PAGE) using a 10% non-SDS-PAGE minigel with a running buffer containing 25 mM Tris-HCl (pH 8.3) and 192 mM glycine at 20 mA for 3 h at 4°C. The activities of CDs were detected by incubating the gel in a buffer containing 100 mM Tris-HCl (pH 8.6), 10 mM EDTA, 50 mM cysteine, 20 μM PLP, and 1.6 mM $BiCl_3$ at room temperature for 2 h.

Measurement of CysM (OASS-B) activity. An overnight culture of each strain in the LB medium was diluted 1:20 in 2 ml of fresh LB medium containing 5 $mg \cdot ml^{-1}$ glucose in a test tube and grown aerobically to an OD_{600} of 2.5 at 30°C. The cells were collected from 1 ml of the culture broth by centrifugation, washed with 100 mM potassium phosphate buffer (pH 7.2), and then suspended in 450 μl of 100 mM potassium phosphate buffer (pH 7.2). The cells were disrupted by ultrasonication, and the supernatant (cell extract) was obtained by centrifugation. The reaction was initiated by adding 40 μl of cell extract to 160 μl of a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.2), 10 mM *O*-acetylserine, and 2 mM $Na_2S_2O_3$ and incubating for 20 min at 34°C; this was then terminated by heating at 65°C for 10 min. Cysteine production was analyzed using the method described by Gaitonde (40). One unit of enzyme activity was defined as 1 μmol cysteine produced per minute per milligram of total protein.

Fermentative production of cysteine. Each strain was streaked onto an LB plate and grown overnight. Cells were collected using a 10-μl loop, which was passed through 7 cm of bacteria on the culture plates and was then inoculated into 2 ml of the fermentation medium in test tubes (23 mm internal diameter by 200 mm length). The cells were incubated at 32°C with agitation until all the sugar was consumed, unless otherwise stated. The quantitative analysis of cysteine in culture was performed according to the method described by Gaitonde (40). Before adding the ninhydrin reagent, samples were reduced by incubation with 10 mM dithioerythritol in 10 mM Tris-HCl buffer (pH 8.5) for 10 min. This assay system allows the determination of cysteine in its oxidized form (cystine) and condensation form with pyruvate (2-methyl-2,4-thiazolidine carboxylic acid) (17, 21). This assay system also allows the determination of *S*-sulfocysteine, a precursor of cysteine in the cysteine biosynthetic pathway. All these derivatives of cysteine are easily converted to cysteine by either simple biological or chemical reaction, and thus, the assay provides an informative output for the evaluation of the ability to produce cysteine as the "total cysteine." Cysteine yield was defined as grams of the final amount of the product obtained from 1 g of glucose expressed as a percentage.

Measurement of CD activity. A 1:25 dilution of an overnight culture of each strain grown in LB medium was inoculated into fermentation medium and grown aerobically to logarithmic phase (4 to 5 h) at 32°C before harvesting by centrifugation. Cells were collected, washed with a buffer containing 0.85% NaCl at 4°C, and suspended in 0.1 M potassium phosphate buffer (pH 8.0) containing 50 $\mu g \cdot ml^{-1}$ bovine serum albumin and 10 μM pyridoxal phosphate. Cells were disrupted by ultrasonication and centrifuged, and the supernatant (cell extract) was analyzed for CD activity, as previously described (54). Total protein concentrations were analyzed using a standard Coomassie protein assay system.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02502-16.

TEXT S1, PDF file, 0.05 MB.

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